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## Bilirubin Influence on Oxidative Lung Damage and Surfactant Surface Tension Properties

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**Summary.** To study the hypothesis that hyperbilirubinemia might reduce in vivo oxidative lung damage while also diminishing lung surfactant surface tension properties during acute lung injury, we performed a randomized study in a rabbit model of acute lung injury. Twenty rabbits were randomized to receive bilirubin or saline intravenously. Acute lung injury was induced by lung lavages with saline. Lung tissue oxidation was evaluated by measuring total hydroperoxide (TH), advanced oxidation protein products (AOPP), and protein carbonyls (PC) in bronchial aspirate (BA) samples. Surface surfactant activity was studied in BA samples using a capillary surfactometer. Bilirubin BA concentration increased in bilirubin-treated rabbits, while it remained undetectable in controls. A similar increase in TH, AOPP, and PC bronchial aspirate concentrations was found in both the study and control groups, while surfactant surface activity was lower in the bilirubin than in the control group. We conclude that during hyperbilirubinemia, bilirubin enters the lung tissue, where it can be detected in BA fluid. Bilirubin is not effective as an antioxidant agent and exerts a detrimental effect on lung surfactant surface tension properties. These findings may have relevance to the management of premature neonates suffering from respiratory distress syndrome and hyperbilirubinemia. *Pediatr Pulmonol.* 2004; 38:179–185. © 2004 Wiley-Liss, Inc.

**Key words:** bilirubin; oxidative stress; surfactant; acute lung injury.

### INTRODUCTION

Hyperbilirubinemia is a frequent complication in newborn infants which generally occurs in the first days of life. During this period, bilirubin can distribute to many tissues.<sup>1</sup> However, the bilirubin toxic effect has been investigated primarily in the central nervous system,<sup>2</sup> while little work has been done to assess the possible effects of bilirubin on other organs, such as the lungs. In fact, in hyperbilirubinemic preterm infants with respiratory distress syndrome (RDS), bilirubin can enter the alveolar space in fluid leaking from capillaries, where its presence at necropsy has been called “yellow hyaline membrane disease.”<sup>3,4</sup> The presence of bilirubin in alveolar spaces might result in associated antioxidant effects or interactions with endogenous surfactant, both of which could be of clinical importance.

Several reports emphasized that the antioxidant potential of bilirubin in neonates is greater than urates,  $\alpha$ -tocopherol, or ascorbates.<sup>5</sup> In fact, although its role in vivo has not been definitively clarified in infants,<sup>6–10</sup> the antioxidant effect of bilirubin as a scavenger of reactive species of oxygen was well-documented in vitro<sup>11–14</sup> as

well as in animal studies.<sup>15</sup> Moreover, bilirubin was demonstrated to impair the surface tension of natural lung surfactant in vitro,<sup>16</sup> its effect being independent of pH.<sup>17</sup>

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Based on the above observations, we hypothesized that, during hyperbilirubinemia, bilirubin might enter the alveolar space and exert a protective antioxidant effect on bronchoalveolar tissues, on the one hand, and a detrimental effect on lung surfactant surface tension properties, on the other. To assess this hypothesis, we performed a prospective, randomized study in which hyperbilirubinemia was induced in a juvenile rabbit model of acute lung injury, and the bilirubin level was measured in serial bronchial aspirate (BA) samples. Lung tissue oxidative damage was investigated measuring total hydroperoxide (TH), advanced oxidation protein products (AOPP), and protein carbonyl (PC) concentrations in BA samples. Surfactant surface activity was studied using the capillary surfactometer method.

## MATERIALS AND METHODS

The experimental protocol was designed in compliance with the recommendations of the European Economic Community (86/609/CEE) for the care and use of laboratory animals, and was approved by the Animal Care Committee of the University of Florence.

### Animals and Instrumentation

Twenty-four juvenile New Zealand rabbits of either sex (age 30–40 days) were anesthetized with ketamine (25 mg/kg intramuscularly) and xylazine (4 mg/kg intramuscularly). Anesthesia was maintained with a continuous infusion of ketamine (10 mg/kg/hr) and xylazine (4 mg/kg/hr). Muscle relaxation was induced by intravenous administration of pancuronium bromide (0.2 mg/kg) and maintained with 0.1 mg/kg doses as needed to control movement.

The anterior neck was dissected, and a tracheostomy was performed. A cuffed endotracheal tube (3.0–3.5 mm internal diameter, Sheridan Catheter, Argyle, USA) was placed in the trachea with the tip proximal to the carina. The endotracheal tube was connected to a continuous-flow, time-cycled, pressure-limited ventilator (Bird 750 PSV, Viasys). Humidification and heating of the respiratory gases were provided with a standard humidifier. Initially the animals were ventilated with a continuous flow of 10 l/min,  $\text{FiO}_2$  1.0, with a peak inspiratory pressure (PIP) of 12 cmH<sub>2</sub>O, a positive end expiratory (PEEP) of 2 cm H<sub>2</sub>O, and a respiratory rate of 25/min.

A carotid artery catheter was inserted for continuous blood pressure monitoring and blood sampling. The catheter was connected to pressure transducers, zero-referenced to midchest level, and a standard limb lead electrocardiogram (ECG) was used. A vascular cannula was inserted into the right internal jugular vein for continuous infusion of fluids (lactate Ringer's solution at the rate of 4 ml/kg/hr) and medications. Rectal temperature was kept within normal range, using electric warming pads.

During the entire experiment, polygeline (Emagel®) and/or dopamine, 10–20 µg/kg/min, were administered as needed to maintain a mean arterial pressure above 65 mmHg. Metabolic acidosis ( $\text{pH} < 7.25$  and  $\text{BE} < 8$  mmol/l) was corrected with sodium bicarbonate or THAM infusion (in case of hypercapnia,  $\text{PaCO}_2 > 45$  mmHg). Arterial blood gases and pH were measured with a blood gas analyzer (Radiometer ABL 610, Copenhagen, Denmark), correcting for body temperature.

The pH,  $\text{pO}_2$ , and  $\text{pCO}_2$  were recorded in each animal before induction of lung injury ( $t_0$ ), and at 15 min ( $t_{15}$ ), 1 hr ( $t_{60}$ ), 2 hr ( $t_{120}$ ), and 4 hr ( $t_{240}$ ) after beginning mechanical ventilation, following induction of lung injury. At the same time of these data recordings, the oxygenation index and ventilation efficient index were computed as previously described<sup>18–20</sup>:  $\text{OI} = [\text{mean airway pressure} \times \text{inspired oxygen fraction} / \text{pO}_2 \text{ (mmHg)} \times 100]$ ;  $\text{VEI} = 3,800 / [\text{respiratory rate (PIP} - \text{PEEP)} + \text{pCO}_2 \text{ (mmHg)}]$ , where VEI relates alveolar ventilation to respirator input and 3,800 is a constant for estimating alveolar ventilation ( $3,800 / \text{pCO}_2$ ), with the assumption of  $\text{CO}_2$  production near normal minimal volumes of  $5 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  in resting animals.

### Experimental Protocol

Thirty minutes after instituting mechanical ventilation, the animals were randomized by coin toss to receive a bilirubin solution or saline only, intravenously. To prepare the solution at a bilirubin concentration of 3.2 mg/ml, bilirubin (crystalline bilirubin, Sigma Chemical Co., St. Louis, MO) was dissolved in 0.5 M NaOH, 0.055 M phosphate, and 5% beef albumin at pH 7.4 (10:20:70).<sup>21</sup> A bilirubin loading dose of 160 mg/kg was administered over 15 min, followed by a maintenance dose of 64 mg/kg/hr until the end of the study. This dose of bilirubin was estimated to result in a plasma bilirubin level which would range from 200–300 µmol/l.<sup>21</sup> Blood samples were obtained before bilirubin or saline infusion had begun ( $T_0$ ), after 1 ( $T_1$ ) and 2 ( $T_2$ ) hr of infusion, and at the end of the study ( $T_3$ ). Total bilirubin plasma level (B<sub>tot</sub>) was measured in blood samples by reflectance spectrophotometry (Microbilimeter<sup>TM</sup>, Ginevri, Rome, Italy). The accuracy of B<sub>tot</sub> measurement in our unit was recently tested, and the correlation between our laboratory method

#### ABBREVIATIONS

|                  |                                     |
|------------------|-------------------------------------|
| AOPP             | Advanced oxidation protein products |
| BA               | Bronchial aspirate                  |
| B <sub>tot</sub> | Total bilirubin plasma level        |
| PC               | Protein carbonyls                   |
| TH               | Total hydroperoxide                 |

and the HPLC Btot measurement was high ( $r=0.927$ ; 95% CI = 0.906–0.944).<sup>22</sup>

Bronchial aspirate bilirubin and total protein BA content were measured at  $T_0$ ,  $T_1$ ,  $T_2$ , and  $T_3$ . Bilirubin concentration was measured by reflectance spectrophotometry (see above), while protein detection was performed with the bicinchoninic acid method.<sup>23</sup>

### Induction of Lung Injury

Immediately after bilirubin and saline infusion had begun, respiratory failure was induced by repeated lung lavages with 30 ml/kg of warm saline.<sup>24,25</sup> Lung lavages were repeated at 3-min intervals until  $pO_2$  was below 100 mmHg at the following ventilator settings:  $FiO_2$ , 1.0; PIP, 20 cm  $H_2O$ ; PEEP, 5 cm  $H_2O$ ; and frequency, 60/min. To achieve this target, 5–6 lavages were required. Then the ventilator setting was adjusted to maintain a  $pO_2$  of 55–80 mmHg and  $pCO_2$  of 45–60 mmHg, to avoid hyperoxia and decrease baro/volutrauma. Maximum ventilator settings included a PIP of 26 cm  $H_2O$ , a PEEP of 5 cm  $H_2O$ , and a respiratory rate of 60 breaths/min, with inspiratory/expiratory (I:E) ratio of 1:2.

Bronchial aspirate samples from animals were taken using the following technique: 1 ml/kg sterile 0.9% saline was instilled with a 10-ml syringe via an 8F gauge feeding catheter placed in the ETT, its length calculated so that its tip would extend 2 cm beyond the distal end of the ETT. The saline was instilled and immediately aspirated back into the syringe. The mean volume of saline returned was 1.2 ml. All samples were clarified by centrifugation, and the supernatant was immediately frozen to  $-70^\circ C$  and kept for subsequent analysis. Bronchial aspirate samples were taken from each animal before lung injury induction and at the start of bilirubin and saline infusion ( $T_0$ ), after 1 hr ( $T_2$ ) and 2 hr ( $T_2$ ) of bilirubin or saline infusion, and at the end ( $T_3$ ) of the study.

### Oxidative Stress Assay

In each bronchial aspirate sample, we measured the TH, AOPP, and PC concentrations. Total hydroperoxide represents a measure of overall oxidative stress, given that it is the intermediate oxidative product of lipids, peptides, and amino acids. Its production was measured with a d-ROMs Kit (Diacron srl, Italy) by the method described by Buonocore et al.<sup>26</sup> This method makes it possible to estimate the total amount of hydroperoxide present in a 20- $\mu$ l sample by using a spectrophotometric procedure. Hydroperoxidic groups were attacked by the iron decomplexed from transport protein in 1 ml of acetate buffer at pH 4.8, to catalyse reactive oxygen metabolite formation by Fenton's reaction. The peroxy and alkoxy radicals produced, whose quantities were directly proportional to peroxides present in the bronchial aspirate, were trapped chemically by 20  $\mu$ l of chromogen (N,N-

diethylpara-phenyl-diamine) in an electron-transfer process leading to the formation of the radical cation of this chromogen. The purple color resulting from this reaction over time was then monitored in a UV-VIS spectrophotometer (Perkin Elmer  $\lambda$  16, Norwalk, CT) at 505 nm. The results were expressed in conventional units (Carr units: the value of 1 Carr unit is equal to a concentration of 0.08 mg/dl of hydrogen peroxide).

Simultaneous determination of AOPP could provide information regarding another aspect of protein involvement in free radical reactions, i.e., oxidized proteins that have lost their oxidant properties. We measured AOPP by the method of Witko-Sarsat et al.,<sup>27</sup> using spectrophotometry on a microplate reader. The AOPP were calibrated with chloramine-T solutions that absorb at 340 nm in the presence of potassium iodide. In test wells, 200  $\mu$ l of bronchial aspirate sample diluted 1:5 in phosphate-buffered saline (PBS) were distributed on a 96-well microtiter plate, and 20  $\mu$ l of acetic acid were added. In standard wells, 10  $\mu$ l of 1.16 M potassium iodide were added to 200  $\mu$ l of chloramine-T solution (0–100  $\mu$ mol/l) followed by 20  $\mu$ l of acetic acid. The absorbency of the reaction mixture was immediately read at 340 nm on the microplate reader against a blank containing 200  $\mu$ l of PBS, 10  $\mu$ l of potassium iodide, and 20  $\mu$ l of acetic acid. Because the absorbency of chloramine-T at 340 nm is linear up to 100 mol/l, AOPP concentrations were expressed as  $\mu$ mol/l chloramine-T equivalents.

Protein carbonyl derives from a variety of oxidative mechanisms that include fragmentation and amine oxidation, and its measurement, therefore, is a sensitive index of oxidative injury.<sup>28</sup> The quantity of PC in a protein sample can be determined by derivatizing with dinitrophenylhydrazine (DNP) and measuring protein-bound DNP with an anti-DNP antibody. Protein carbonyl concentration was determined by enzyme-linked immunosorbent assay (ELISA), using the Zentech PC Test Kit (Zenith Technology, Dunedin, New Zealand), by the method of Buss et al.<sup>28</sup> The ELISA method enables PC to be measured quantitatively with microgram quantities of protein. The assay is set up so that about 1  $\mu$ g of derivatized protein is applied to each well of the ELISA plate. Proteins from any sample are incubated at room temperature with DNP for 45 min and nonspecifically adsorbed to an ELISA plate by incubating overnight at  $4^\circ C$ . Proteins are probed with biotinylated anti-DNP antibody followed by streptavidin-linked horseradish peroxidase. Finally, the chromatin reagent containing peroxidase is added, and the peroxidase catalyses the oxidation of tetra-methyl-benzidine (TMB). The reaction is stopped by adding acid, and absorbance is measured for each well at 450 nm.

To establish intra- and interassay accuracy, multiple measurements were performed the same day and for a whole month. TH was within 12%, AOPP was within 10%, and PC precision was within 9%.

## Surface Activity of Surfactant

Surface activity of surfactant was studied using the capillary surfactometer, as described by Lema et al.<sup>29</sup> This technique evaluates the ability of surfactant to maintain the patency of a narrow tube. The instrument uses a glass capillary to simulate a terminal conducting airway. In a short section, the capillary is constricted (inner diameter, 0.3 mm) to a width similar to that of a human respiratory bronchiole. Liquid tends to accumulate in this narrow section, and when a small amount (0.5  $\mu$ l) of the liquid to be evaluated is instilled into the constricted section, it remains there, but will be extruded when pressure, continuously recorded, is slowly raised at one end of the capillary. This will cause the blocking liquid to be pushed away from the narrow section. If it contains well-functioning pulmonary surfactant, a film will quickly form at the air-liquid interface, and by exerting high surface pressure, it will prevent the liquid from returning to the narrow section of the capillary. Consequently, a steady flow of air through the capillary will meet no resistance, and a pressure of zero is recorded during the entire 120-sec period of pressure recording that follows the initial extrusion of the liquid. Thus, the capillary is open 100% of the recording time. If, on the other hand, the surfactant is inadequate in quantity or quality, the liquid will return once or repeatedly. The continuous airflow will meet resistance, and consequently, pressure will not remain at zero during the entire period of recording (120 sec). A minicomputer calculates and prints the percentage of time that pressure remained at zero and expresses the surface tension properties of surfactant as open in %. Each sample was studied five times with a capillary surfactometer.

## Tissue Removal and Pathologic Examination

At the end of the experiment, the animals were killed with an overdose of pentobarbital. The thorax was carefully opened to check for signs of pneumothorax and to harvest tissue. The trachea and lungs were removed and put in a buffered 10% formalin bath for 24 hr. Two random pulmonary specimens were obtained from the upper and lower lobes of both lungs. Routine techniques were used to prepare the tissues for paraffin embedding. Five-micrometer-thick sections were stained with hematoxylin-eosin, and two pathologists performed the blinded microscopic examination. Terminal airways distension was graded 0 when it was similar to that of control lungs, 1 when dilated (between 1–1.5 times with respect to the control airway caliber), and 2 when very dilated (more than 1.5 times with respect to the control airway caliber). In addition, the following histological features were analyzed: areas of atelectasis, interstitial and/or alveolar edema, inflammatory infiltration, interlobular septa ruptures, and desquamation of bronchiolar epithelium. Each

pathological feature was evaluated as 1 when present and 0 when absent. A pulmonary damage score (negative = 0, slight = 0–1, moderate = 2–3, and severe = 4) was achieved by adding up the score.

## Statistical Analysis

In planning our study, we calculated that a sample size of at least 10 animals in each group would be required to detect a difference of 50% in TH bronchial aspirate concentration in the bilirubin group in comparison to the control group, with 80% power at a 0.05 alpha level.

Results are expressed as mean  $\pm$  SD. The data were analyzed for statistically significant differences by ANOVA test within groups and by Student's *t*-test between groups. Simple regression analysis was used to assess the correlation in bilirubin-treated animals between Btot and bilirubin level in BA samples.

## RESULTS

Four animals died during the saline-lavage phase before randomization, and they were consequently excluded from further data analysis. Among the remaining animals, 10 rabbits were randomized to receive bilirubin infusion and 10 to receive saline infusion. Body weight ( $1.98 \pm 0.10$  vs.  $2.02 \pm 0.18$  kg) and the number of saline lavages ( $6 \pm 1$  vs.  $6 \pm 1$  times) did not differ significantly between groups. The animals received  $10 (\pm 22)$  ml/kg polygeline and  $2.9 (\pm 1.8)$  mmol/kg of THAM without significant differences between the bilirubin-treated ( $11 \pm 17$  ml/kg;  $2.8 \pm 2.1$  mmol/kg) and untreated ( $9 \pm 22$  ml/kg;  $3.2 \pm 1.5$  mmol/kg) rabbits.

## Gas Exchange

Fifteen minutes after induction of lung injury ( $t_{15}$ ), we observed a significant decrease of pH and  $pO_2$  and an increase of  $pCO_2$ . The values of pH,  $pO_2$ , and  $pCO_2$  stabilized at  $t_{60}$  and were substantially unchanged at  $t_{120}$  and  $t_{240}$ . The oxygenation index increased and VEI decreased at  $t_{15}$ . Then, OI and VEI improved at  $t_{60}$  and did not vary at  $t_{120}$  and at  $t_{240}$ . The values of pH,  $pO_2$ ,  $pCO_2$ , OI, and VEI did not significantly differ between groups.

## Bilirubin and Oxidative Stress Assays

Total bilirubin plasma levels were  $3.5 \pm 0.3$ ,  $288.9 \pm 22.5$ ,  $255.2 \pm 25.1$ , and  $268.2 \pm 23.1$   $\mu$ mol/l at  $T_0$ ,  $T_1$ ,  $T_2$ , and  $T_3$  in the bilirubin-treated animals, while the mean value in the control group was  $3.6 \pm 0.4$   $\mu$ mol/l (Table 1). In bilirubin-infused animals, the bilirubin BA concentration increased from 0 at  $T_0$  to  $2.5 \pm 0.2$  at  $T_1$ ,  $2.4 \pm 0.4$  at  $T_2$ , and  $2.6 \pm 0.4$   $\mu$ mol/l at  $T_3$ , while it remained undetectable in BA samples of control animals (Table 1). The total protein content of BA samples was similar in the two groups and ranged from  $3.5 \pm 0.5$  to  $4.1 \pm 0.7$  mg/dl

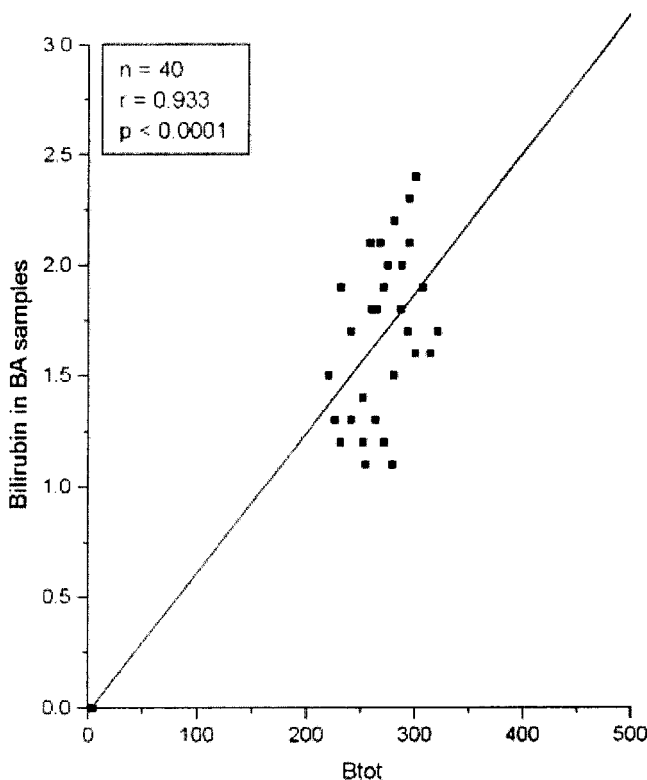
**TABLE 1—Measurements of Bilirubin Plasma Level ( $\mu\text{mol/l}$ ), Bilirubin Levels in BA Samples ( $\mu\text{mol/l}$ ), and Total Protein in BA Samples ( $\text{mg/dl}$ ) in Bilirubin-Treated and Control Animals Before ( $T_0$ ), After 1 ( $T_1$ ) and 2 hr ( $T_2$ ) of Bilirubin Infusion, and at End ( $T_3$ ) of Study Period: mean  $\pm$  SD**

|       | Bilirubin plasma levels |               | Bilirubin levels in BA samples |          | Total protein levels in BA samples |               |
|-------|-------------------------|---------------|--------------------------------|----------|------------------------------------|---------------|
|       | Bilirubin               | Controls      | Bilirubin                      | Controls | Bilirubin                          | Controls      |
| $T_0$ | $3.5 \pm 0.3$           | $3.1 \pm 0.4$ | 0                              | 0        | $3.6 \pm 0.6$                      | $3.5 \pm 0.5$ |
| $T_1$ | $288.9 \pm 22.5^*$      | $4.2 \pm 0.3$ | $2.5 \pm 0.2^*$                | 0        | $4.1 \pm 0.7$                      | $3.9 \pm 0.6$ |
| $T_2$ | $255.2 \pm 25.1^*$      | $3.2 \pm 0.6$ | $2.4 \pm 0.4^*$                | 0        | $3.9 \pm 0.4$                      | $3.8 \pm 0.5$ |
| $T_3$ | $268.2 \pm 23.1^*$      | $3.8 \pm 0.4$ | $2.6 \pm 0.4^*$                | 0        | $3.8 \pm 0.5$                      | $4.0 \pm 0.4$ |

\* $P < 0.0001$  vs.  $T_0$  and vs. control group.

(Table 1). A statistically significant correlation was observed between Btot and bilirubin level in BA samples ( $r = 0.933$ ,  $P < 0.0001$ ; Fig. 1).

During the study, we found a significant increase of TH, AOPP, and PC bronchial aspirate concentrations at  $T_3$  in both groups, without differences between groups (Table 2). The mean increase of TH from  $T_0$  to  $T_3$  was  $21.7 \pm 6.5\%$  in the bilirubin group and  $15.1 \pm 3.5\%$  in the control group. The mean increase of AOPP from  $T_0$  to  $T_3$  was  $53.8 \pm 25.0\%$  in the bilirubin group and  $40.2 \pm 20.1\%$  in the control group. The mean increase of PC from  $T_0$  to  $T_3$  was  $50.2 \pm 20.3\%$  in the bilirubin group and  $32.3 \pm 16.7\%$  in the control group.



**Fig. 1. Correlations between Btot ( $\mu\text{mol/l}$ ) and bilirubin concentration in BA samples ( $\mu\text{mol/l}$ ) in bilirubin-treated animals.**

### Surface Activity of Surfactant

Surfactant surface activity declined after saline lavage in both groups, but at the end of the study period it was lower in the bilirubin group than in the control group ( $32.1 \pm 2.8$  vs.  $47.7 \pm 4.9$  open in %; Fig. 2).

### Macro-Microscopic Features

On gross examination, the lungs of both groups were sufficiently aerated except for some areas of atelectasis, particularly in the lower lobes. On microscopic examination, all pulmonary specimens showed scattered areas of collapsed alveolar walls, and some alveoli contained macrophages. Moreover, distal airways appeared moderately dilated, and some interlobular septa leakages were observed. These features were confirmed by the pulmonary damage score, which was  $2.4 \pm 0.6$  and  $2.6 \pm 0.5$  in the bilirubin and control groups, respectively, reflecting moderately pathologic lung damage, but without a significant difference noted between groups ( $P > 0.05$ ).

### DISCUSSION

Our results demonstrate that in the hyperbilirubinemic juvenile rabbit model of acute lung injury, bilirubin is able to enter the alveolar space during hyperbilirubinemia, akin to its entry into lung tissue in preterm infants during RDS,<sup>3,4</sup> and that the bilirubin level in BA samples is directly correlated with Btot.

Our study did not demonstrate any protective effects of bilirubin in vivo against oxidative lung injury. In fact, during mechanical ventilation, the degree of lung tissue oxidation was similar in the bilirubin-treated and untreated animals, as evidenced by the similar changes of TH, AOPP, and PC in BA samples.

There has been some inconsistency with regard to the effects of bilirubin as antioxidant. Our results seem to disagree with previous studies using in vitro<sup>11–14</sup> and animal models,<sup>15</sup> both of which demonstrated the antioxidant properties of bilirubin. However, the in vitro studies, although very accurate, do not parallel what

**TABLE 2—Measurements of TH (U Carr/L), AOPP ( $\mu\text{mol/l}$ ), and PC Levels (nmol/mg of Proteins) in Bilirubin-Treated and Control Animals Before ( $T_0$ ), After 1 ( $T_1$ ) and 2 hr ( $T_2$ ) of Bilirubin Infusion, and at End ( $T_3$ ) of Study Period: mean  $\pm$  SD**

|       | TH              |                  | AOPP           |                | PC               |                 |
|-------|-----------------|------------------|----------------|----------------|------------------|-----------------|
|       | Bilirubin       | Controls         | Bilirubin      | Controls       | Bilirubin        | Controls        |
| $T_0$ | 21.2 $\pm$ 1.3  | 21.2 $\pm$ 3.1   | 1.3 $\pm$ 0.4  | 1.5 $\pm$ 0.3  | 0.8 $\pm$ 0.5    | 0.9 $\pm$ 0.2   |
| $T_1$ | 22.8 $\pm$ 1.5  | 21.0 $\pm$ 4.1   | 1.3 $\pm$ 0.8  | 1.2 $\pm$ 0.3  | 1.1 $\pm$ 0.2    | 1.0 $\pm$ 0.3   |
| $T_2$ | 21.8 $\pm$ 3.5  | 22.4 $\pm$ 4.3   | 1.2 $\pm$ 0.4  | 1.2 $\pm$ 0.6  | 1.2 $\pm$ 0.3    | 1.5 $\pm$ 0.4** |
| $T_3$ | 25.8 $\pm$ 3.8* | 24.4 $\pm$ 2.9** | 2.0 $\pm$ 1.4* | 2.1 $\pm$ 0.3* | 1.2 $\pm$ 0.3*** | 1.2 $\pm$ 0.3** |

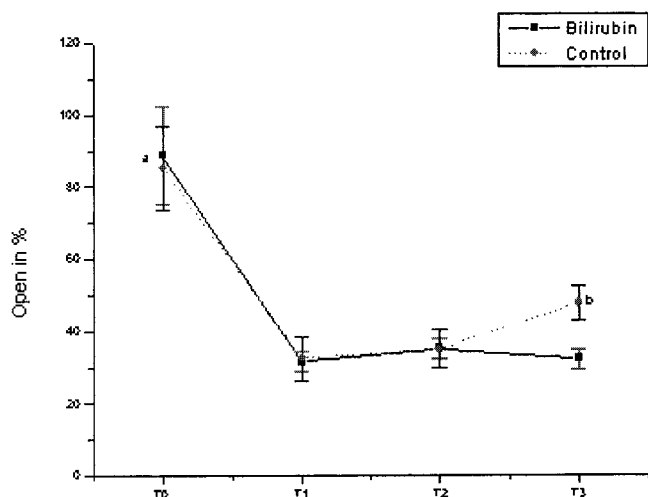
\* $P < 0.05$  vs.  $T_0$ ,  $T_1$ , and  $T_2$ .\*\* $P < 0.05$  vs.  $T_0$  and  $T_1$ .\*\*\* $P < 0.05$  vs.  $T_0$ .

actually occurs in vivo. In the study of Dennerly et al. performed on Gunn rats, an antioxidant effect of bilirubin was found only in the circulatory compartment, where bilirubin has the highest concentration.<sup>15</sup> In fact, and consistent with our findings, in that same study, jaundiced animals exposed to hyperoxia exhibited a lung content of thiobarbituric acid-reactive substances and conjugated dienes similar to that of nonjaundiced animals, indicating a similar degree of oxidative lung damage.<sup>15</sup> Those and now our own data indicate that bilirubin, despite its ability to cause a systemic antioxidant effect, does not appear able to exert a localized antioxidant effect in lung tissue. One possible explanation for this phenomenon is that the concentration of bilirubin in lung tissues (and indeed in the alveolar space) is too low to let it exert its antioxidant properties.<sup>15</sup> Moreover, our study was performed in a juvenile rabbit model, and we cannot rule out that the antioxidant role of bilirubin could be more important in a

premature neonatal model where endogenous levels of other antioxidants are deficient.

On the other hand, other studies in vitro<sup>13</sup> and in vivo<sup>30</sup> showed that bilirubin is ineffective in preventing oxidative damage in some conditions: Mireles et al. demonstrated in vitro that after oxidative stress, a bilirubin concentration  $>30$  mg/dl is associated with an increase in protein oxidation;<sup>13</sup> Pauly et al. found that an infusion of 15 mg/kg of bilirubin in infant piglets was not effective in preventing the oxidative stress and pulmonary hypertension induced by group B streptococcus.<sup>30</sup> Furthermore, previous studies in preterm infants showed controversial results with regard to the possible systemic antioxidant properties of bilirubin. In particular, Yigit et al.,<sup>6</sup> Gopinathan et al.,<sup>7</sup> and Dani et al.<sup>10</sup> did not find a correlation between serum bilirubin and plasma antioxidant capacity in preterm infants, while this correlation was found by Belanger et al.<sup>8</sup> and Hammerman et al.<sup>9</sup>

In our study, we also investigated the effect of hyperbilirubinemia on surfactant surface activity, and we found that at the end of the study period, activity was significantly lower in the bilirubin-treated group than in the control group. Thus, in our model of lung injury, the increase of bilirubin plasma level induces the appearance of bilirubin in bronchoalveolar fluid, which in turn causes a partial inhibition of surfactant property of lowering surface tension. This is the first in vivo observation of the detrimental effect of bilirubin on surfactant surface activity, although similar findings were described in vitro by Amato et al.<sup>16,17</sup> They demonstrated that bilirubin impairs the surface tension activity of porcine lipid extract surfactant but does not affect synthetic surfactant activity,<sup>16</sup> and that this effect occurs independent of pH.<sup>17</sup> The different effect of bilirubin on natural and synthetic surfactant was attributed to the different composition of the two surfactants: it is possible that tyloxapol, an alcohol that is contained in the synthetic surfactant, can "neutralize" the adverse effect of bilirubin, but the most attractive hypothesis is that bilirubin can interact with the surfactant proteins SP-B or SP-C, which are absent in synthetic surfactant.



**Fig. 2.** Changes of surfactant surface activity (open in %) in bilirubin and control groups before lung injury induction ( $T_0$ ), at 1 hr ( $T_1$ ) and 2 hr ( $T_2$ ) after beginning of bilirubin infusion, and at end ( $T_3$ ) of study period. Data are shown as mean  $\pm$  SD. <sup>a</sup> $P < 0.05$ ,  $T_0$  vs.  $T_1$ ,  $T_2$ , and  $T_3$ . <sup>b</sup> $P < 0.05$   $T_3$  vs.  $T_2$  and  $T_1$  in control group and  $T_3$  in control group vs.  $T_3$  in bilirubin group.

Thus, our *in vivo* results, which confirm the observations of Amato et al.,<sup>16,17</sup> might have practical implications for the management of jaundiced infants affected by respiratory distress syndrome requiring exogenous surfactant treatment. It is possible that more intense treatment of hyperbilirubinemia, or a more aggressive treatment with exogenous surfactant, may be useful in the treatment of jaundiced infants with respiratory distress syndrome.

In conclusion, we demonstrated that in this animal model of acute lung injury, hyperbilirubinemia induced by the intravenous injection of bilirubin results in the presence of bilirubin in BA fluid. This bilirubin is not effective as an antioxidant agent and exerts a detrimental effect on the surface tension properties of lung surfactant. These results suggest the opportunity of investigating the possible correlation between hyperbilirubinemia and the severity of respiratory distress syndrome in jaundiced newborn infants with respiratory failure.

## REFERENCES

- Bloomer JR, Berk PD, Vergalla J, Berlin NI. Influence of albumin on the extravascular distribution of bilirubin. *Clin Sci Mol Med* 1973;45:517–522.
- Cashore WJ. The neurotoxicity of bilirubin. *Clin Perinatol* 1990;17:437–447.
- Valdoes Dapena MA, Nissim JE, Arey JB, Godleski J, Schaaf HD, Haust MD. Yellow pulmonary hyaline membrane. *J Pediatr* 1976; 89:128–132.
- Morgenstern B, Klionsky B, Doshi N. Yellow hyaline membrane disease. Identification of the pigment and bilirubin binding. *Lab Invest* 1981;44:514–518.
- Miller NJ, Rice-Evans C, Davies MJ, Gopinathan V, Milner A. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clin Sci* 1993;84:407–412.
- Yigit S, Yurdakok M, Kilinc K, Oran O, Erdem G, Tekinalp G. Serum malondialdehyde concentrations in babies with hyperbilirubinemia. *Arch Dis Child Fetal Neonatal Ed* 1999;80:235–237.
- Gopinathan V, Miller NJ, Milner AD, Rice Evans CA. Bilirubin and ascorbate antioxidant activity in neonatal plasma. *FEBS Lett* 1994;349:197–200.
- Bellanger S, Lavole JC, Chessex P. Influence of bilirubin on the antioxidant capacity of plasma in newborn infants. *Biol Neonate* 1997;71:233–238.
- Hammerman C, Goldstein R, Kaplan M, Eran M, Goldschmidt D, Eidelman AI, Gartner LM. Bilirubin in the premature: toxic waste or natural defense? *Clin Chem* 1998;44:2551–2553.
- Dani C, Martelli E, Bertini G, Pezzati M, Filippi L, Rossetti M, Rizzati G, Rubaltelli FF. Bilirubin plasma level and oxidative stress in preterm infants. *Arch Dis Child Fetal Neonatal Ed* 2003; 88:119–123.
- Wu TW, Wu J, Li RK, Mickle D, Carey D. Albumin-bound bilirubin protects human ventricular myocytes oxyradical damage. *Biochem Cell Biol* 1991;69:683–688.
- Stocker R, Yamamoto Y, McDonagh AF, Spitz DR, Glazer AN, Ames BN. Bilirubin is an antioxidant of possible physiological importance. *Science* 1987;235:1043–1046.
- Mireles LC, Lum MA, Dennerly PA. Antioxidant and cytotoxic effects of bilirubin on neonatal erythrocytes. *Pediatr Res* 1999;45: 355–362.
- Stocker R, Glazer AN, Ames BN. Antioxidant activity of albumin-bound bilirubin. *Proc Natl Acad Sci USA* 1987;84: 5918–5922.
- Dennerly PA, McDonagh AF, Spitz DR, Rogers PA, Stevenson DK. Hyperbilirubinemia results in reduced oxidative injury in neonatal Gunn rat exposed to hyperoxia. *Free Radic Biol Med* 1995;19:395–404.
- Amato M, Schurch S, Grunder R, Bachofen H, Burri PH. Influence of bilirubin on surface tension properties of lung surfactant. *Arch Dis Child* 1996;75:191–196.
- Amato M, Schurch S, Grunder R, Bachofen H, Burri PH. Effects of acidosis on bilirubin-lipid extract surfactant interaction. *Biol Neonate* 1995;68:301–307.
- Al-Rahmani A, Awad K, Miller TF, Wolfson MR, Shaffer TH. Effects of partial liquid ventilation with perfluorodecalin in the juvenile rabbit lung after saline injury. *Crit Care Med* 2000;28: 1459–1464.
- Boynton BR, Carlo WA, Jobe AH. New therapies for neonatal respiratory failure. New York: Cambridge University Press; 1994. p 185.
- Notterh RH, Egan EA, Kwong MS, Holm BA, Shapiro DI. Lung surfactant replacement in premature lambs with extracted lipid from bovine lung lavage: effect of dose, dispersion technique, and gestational age. *Pediatr Res* 1985;19:569–577.
- Roger C, Kozziel V, Vert P, Nehlig A. Effects of bilirubin infusion on local cerebral glucose utilization in the immature rat. *Dev Brain Res* 1993;19:115–130.
- Rubaltelli FF, Glenn RG, Loskamp N, Modi N, Roth-Kleiner M, Sender A, Vert P. Transcutaneous bilirubin measurement: a multicenter evaluation of a new device. *Pediatrics* 2001;107: 1264–1271.
- Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. Measurement of protein using bicinchoninic acid. *Anal Biochem* 1985;150:76–85.
- Lachman B, Robertson B, Vogel J. *In vivo* lung lavage as an experimental model of the respiratory distress syndrome. *Acta Anaesthesiol Scand* 1980;24:231–236.
- Rotta AT, Gunnarsson B, Fuhrman BP, Hernan LJ, Steinhorn DM. Comparison of lung protective ventilation strategy in a rabbit model of acute injury. *Crit Care Med* 2001;29:2176–2184.
- Buonocore G, Perrone S, Longini M, Terzuoli L, Bracci R. Total hydroperoxide and advanced oxidation protein products in preterm hypoxic babies. *Pediatr Res* 2000;47:221–224.
- Witko-Sarsat V, Frielander M, Capeillere-Blandin C, Nguyen-Khoa T, Thu Nguyen A, Zingraff J, Jungers P, Drueke T, Descamps-Latsca B. Advanced oxidation protein products as a novel marker of oxidative stress in uremia. *Kidney Int* 1998;49:1304–1313.
- Buss H, Chan TP, Sluis KB, Domigan NM, Winterbourn CC. Protein carbonyl measurement by a sensitive ELISA method. *Free Radic Biol Med* 1997;23:361–366.
- Lema G, Dryja D, Vargas I, Enhornring G. *Pseudomonas aeruginosa* from patients with cystic fibrosis affects function of pulmonary surfactant. *Pediatr Res* 2000;47:121–126.
- Pauly TH, Smith M, Gillespie M. Bilirubin as an antioxidant: effect of non group B streptococci-induced pulmonary hypertension in infant piglets. *Biol Neonate* 1991;60:320–326.